

# Inactivation of mammalian Ero1 $\alpha$ is catalysed by specific protein disulfide-isomerases

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Disulfide formation within the endoplasmic reticulum is a complex process requiring a disulfide exchange protein such as PDI (protein disulfide-isomerase) and a mechanism to form disulfides *de novo*. In mammalian cells, the major pathway for *de novo* disulfide formation involves the enzyme Ero1 $\alpha$  (endoplasmic reticulum oxidase 1 $\alpha$ ) which couples oxidation of thiols to the reduction of molecular oxygen to form hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). Ero1 $\alpha$  activity is tightly regulated by a mechanism that requires the formation of regulatory disulfides. These regulatory disulfides are reduced to activate and reform to inactivate the enzyme. To investigate the mechanism of inactivation we analysed regulatory disulfide formation in the presence of various oxidants under controlled oxygen concentration. Neither molecular oxygen nor H<sub>2</sub>O<sub>2</sub> was able to

oxidize Ero1 $\alpha$  efficiently to form the correct regulatory disulfides. However, specific members of the PDI family, such as PDI or ERp46 (endoplasmic reticulum-resident protein 46), were able to catalyse this process. Further studies showed that both active sites of PDI contribute to the formation of regulatory disulfides in Ero1 $\alpha$  and that the PDI substrate-binding domain is crucial to allow electron transfer between the two enzymes. The results of the present study demonstrate a simple feedback mechanism of regulation of mammalian Ero1 $\alpha$  involving its primary substrate.

**Key words:** disulfide formation, endoplasmic reticulum, endoplasmic reticulum oxidase 1 (Ero1), protein disulfide isomerase (PDI), protein folding.

## INTRODUCTION

The formation of disulfides within proteins entering the secretory pathway is catalysed by the PDI (protein disulfide-isomerase) family of disulfide exchange proteins [1]. These enzymes act as intermediaries, transferring disulfides from oxidases, such as Ero1 [ER (endoplasmic reticulum) oxidase 1] or peroxiredoxin IV, to substrate proteins [2]. There are two isoforms of the flavoprotein Ero1 ( $\alpha$  and  $\beta$ ) in mammalian cells capable of coupling the reduction of oxygen to the formation of a disulfide and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) [3]. Both isoforms are regulated by a mechanism that involves the formation of disulfides between catalytic and non-catalytic cysteine side chains [4,5]. For the enzyme to become active these regulatory disulfides need to be broken. The regulatory disulfides then reform, thereby inactivating the enzyme and preventing any excess formation of potentially damaging H<sub>2</sub>O<sub>2</sub>.

Ero1 $\alpha$  contains two active site cysteine pairs: a shuttle cysteine pair positioned within an extended flexible loop and an inner active site cysteine pair adjacent to the FAD cofactor [6]. The Ero1 $\alpha$  shuttle cysteines (Cys<sup>94</sup> and Cys<sup>99</sup>) are inactivated by the formation of two regulatory disulfides (Cys<sup>94</sup>–Cys<sup>131</sup> and Cys<sup>99</sup>–Cys<sup>104</sup>) [4–6]. The equivalent shuttle cysteine pair in Ero1 $\beta$  (Cys<sup>90</sup> and Cys<sup>95</sup>) is inactivated by the formation of a disulfide between Cys<sup>90</sup> and Cys<sup>130</sup>. The activities of the two isoforms of Ero1 are very similar as is their mechanism of interaction with PDI so the basis for regulation is likely to be similar.

The initial reduction of the Ero1 regulatory disulfides is thought to be brought about by the action of the primary substrate PDI [6,7]. The interaction between Ero1 and PDI has been shown to be mediated through the PDI substrate-binding domain (the b' domain) [6] with Ero1 preferentially oxidizing the

second catalytic domain within PDI (the a' domain) [7,8]. The intracellular concentration of PDI seems to determine the extent of Ero1 activation [4] giving rise to the suggestion that PDI acts as a control regulator of redox homeostasis in the ER. If the redox conditions become too reducing then reduced PDI activates Ero1 to restore the redox poise. A more oxidizing ER would result in PDI becoming more oxidizing thereby preventing Ero1 activation.

Studies with the yeast enzyme (Ero1p) have shown a similar mechanism of regulation, although the regulatory disulfides do not involve the catalytic cysteine pairs [9]. Rather the breaking and formation of the regulatory disulfides in Ero1p have been suggested to release or restrict the movement of the flexible loop containing the shuttle cysteine pair [9]. The mechanism of reduction of the regulatory disulfide is thought to involve Pdi1p with the reformation of the inactivating disulfide occurring via an autonomous reaction that can be accelerated by oxidized Pdi1p [10]. Hence, the general mechanism of regulation of yeast and mammalian Ero1 appears to be conserved.

Although Ero1 $\alpha$  activity is known to be regulated by intramolecular disulfide bonds, the source of the oxidizing equivalents which inactivate Ero1 $\alpha$  activity has not yet been elucidated. In the present study we tested the hypothesis that Ero1 $\alpha$  is regulated by one or more of three mechanisms: (i) Ero1 $\alpha$  could generate and distribute disulfide bonds intra- or inter-molecularly via the shuttle cysteines to the regulatory cysteine pairs; (ii) H<sub>2</sub>O<sub>2</sub> could induce sulfenylation and disulfide bond formation within the regulatory cysteines; and (iii) that members of the PDI family could directly oxidize Ero1 $\alpha$ . The redox state of a recombinant version of Ero1 $\alpha$  was monitored *in vitro* with a number of potential oxidants to identify the most efficient regulatory mechanism. We report that specific members of the PDI family act to rapidly oxidize and shut down Ero1 $\alpha$  activity,

Abbreviations: AMS, 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid; ER, endoplasmic reticulum; Ero1, ER oxidase 1; ERp46, ER-resident protein 46; NEM, N-ethylmaleimide; PDI, protein disulfide-isomerase; PDIr, PDI-related protein.

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providing further insight into the complicated redox balance within the ER. Further investigation of the PDI–Ero1 $\alpha$  interaction reveals that oxidation of Ero1 $\alpha$  by PDI is dependent on the substrate binding site within the b' domain.

## EXPERIMENTAL

### Recombinant protein expression and purification

The expression and purification of thioredoxin, Ero1 $\alpha$  and PDI wild-type have been described previously [5]. The PDI substrate-binding mutant (I272A, D346A, D348A) and the active site mutants  $\Delta$ S1 and  $\Delta$ S2 were gifts from Professor Lloyd Ruddock (University of Oulu, Oulu, Finland). The proteins were expressed and purified as described for the wild-type protein.

### Western blotting

Following electrophoresis, proteins were transferred on to nitrocellulose membranes (LI-COR Biosciences), which were then blocked for 1 h in 3 % (w/v) non-fat dried skimmed milk powder in 10 mM Tris/HCl buffer (pH 7.5), containing 150 mM NaCl and 0.1 % Tween 20. Blots were then incubated for 1 h with an antibody specific for Ero1 $\alpha$  (Cell Signalling Technologies). Detection was by LI-COR IRDye fluorescent secondary antibodies typically at a 1:5000 dilution. Blots were scanned using an Odyssey Sa Imaging System (LI-COR Biosciences).

### Ero1 $\alpha$ thioredoxin assay

Reduced thioredoxin was prepared following treatment with 10 mM DTT for 10 min at 4°C. The DTT was removed using a PD10 column (GE Healthcare) according to the manufacturer's protocol. Typically for the time course assays, 2  $\mu$ M Ero1 $\alpha$  was incubated with 100  $\mu$ M reduced thioredoxin over 1800 s with samples taken at specific time points. The reactions were stopped by adding SDS sample buffer [200 mM Tris/HCl buffer (pH 6.8), containing 3 % SDS, 10 % glycerol, 1 mM EDTA and 0.004 % Bromophenol Blue] with either 50 mM NEM (*N*-ethylmaleimide) or 20 mM AMS (4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid) to freeze the redox state. Samples were then analysed by SDS/PAGE to visualize the Ero1 $\alpha$  and thioredoxin redox state. For the anaerobic assays, all reagents and buffers were kept in an anaerobic chamber (Coy Laboratory Products) overnight to eliminate oxygen. The assay buffer [50 mM Tris/HCl (pH 7.5) containing 1 mM EDTA] was supplemented with 200  $\mu$ M free FAD as indicated and was purged with nitrogen before incubation in the dark in the anaerobic chamber. There was no significant reduction of FAD during this incubation period.

### Ero1 $\alpha$ re-oxidation assay

All Ero1 $\alpha$  re-oxidation assays were carried out under anaerobic conditions in an anaerobic glove box. Reduced Ero1 $\alpha$  was prepared following incubation with 10 mM DTT for 1 min before applying to a micro Bio-Spin 6 chromatography column (Bio-Rad Laboratories) which was pre-equilibrated in anaerobic buffer [50 mM Tris/HCl (pH 7.5) and 1 mM EDTA] to remove DTT. For the oxygen titration time course assay, the reduced Ero1 samples were incubated with combinations of anaerobic and air-saturated buffer (approximately 250  $\mu$ M oxygen) containing 50 mM Tris/HCl (pH 7.5) and 1 mM EDTA to a final oxygen concentration of 50  $\mu$ M and 225  $\mu$ M. Following incubation, the

reactions were stopped at various times (0, 10, 30, 60 and 300 s) by adding SDS sample buffer [200 mM Tris/HCl buffer (pH 6.8), 3 % SDS, 10 % glycerol, 1 mM EDTA and 0.004 % Bromophenol Blue] containing 25 mM NEM. Samples were analysed by SDS/PAGE under non-reducing conditions and Ero1 $\alpha$  visualized by silver staining.

For the oxidoreductase time course assays, PDI, ERp46 (ER-resident protein 46) or PDIr (PDI-related protein) were oxidized by incubation with 20 mM GSSG for 15 min before applying to Micro Bio-Spin columns to remove GSSG. The oxidized proteins were incubated with 2  $\mu$ M reduced Ero1 $\alpha$  in an anaerobic buffer [50 mM Tris/HCl (pH 7.5) and 1 mM EDTA]. Samples were taken at time points 0, 10, 30, 60 and 300 s and immediately alkylated in SDS sample buffer containing 25 mM NEM. Samples were analysed by SDS/PAGE and visualized by Western blotting.

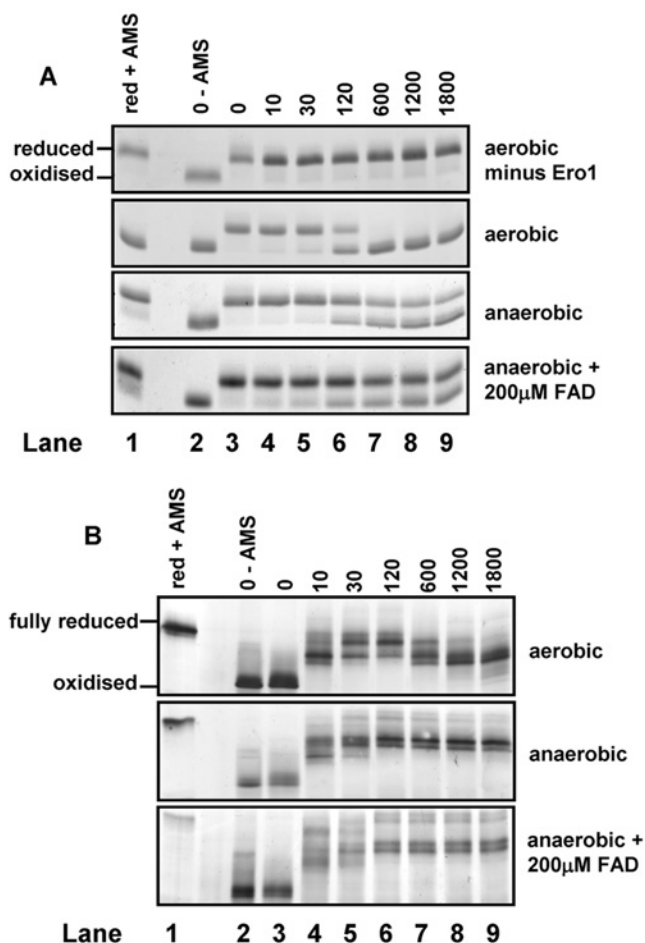
For the H<sub>2</sub>O<sub>2</sub>, GSSG and thioredoxin assays each reagent was incubated at the indicated concentrations with 2  $\mu$ M reduced Ero1 $\alpha$ . Samples were taken at 0, 10, 30, 60 and 300 s, followed by alkylation in SDS sample buffer containing 25 mM NEM. Samples were analysed by SDS/PAGE and visualized by silver staining. Densitometry was then performed on scanned gels using unmodified output images and ImageJ (NIH).

## RESULTS

### Formation of disulfides in Ero1 $\alpha$ is prevented under anaerobic conditions

To establish an assay for the re-oxidation of the regulatory disulfides in Ero1 $\alpha$ , we incubated Ero1 $\alpha$  with a model substrate, thioredoxin under both aerobic and anaerobic conditions. At various time points after initiation of the reaction, samples were alkylated with AMS, which adds ~510 Da per modified thiol, before separation by SDS/PAGE (Figure 1). The oxidized forms of thioredoxin and Ero1 $\alpha$  migrate comparatively quickly through the gel due to their decreased hydrodynamic radius and mass compared with the reduced forms which react with AMS. In the absence of Ero1 $\alpha$  and under aerobic conditions, thioredoxin remains in the reduced slow-migrating form (Figure 1A, top panel) throughout the time course. When Ero1 $\alpha$  was included under aerobic conditions, the redox state of thioredoxin shifts completely from the slower-migrating reduced form, witnessed at 0 and 10 s, to the oxidized form between 120 and 600 s (Figure 1A, upper middle panel). When the assay was carried out under anaerobic conditions, the oxidation of thioredoxin was dramatically inhibited (Figure 1A, lower middle panel), confirming the oxygen dependence of the catalytic activity of Ero1 $\alpha$ . Furthermore, we assessed the ability of exogenous FAD to act as an alternative terminal electron acceptor for Ero1 $\alpha$ . Our findings show that the inclusion of exogenous FAD in this assay does not restore Ero1 $\alpha$  activity, as oxidation of thioredoxin was not increased in the presence of FAD (Figure 1A, bottom panel).

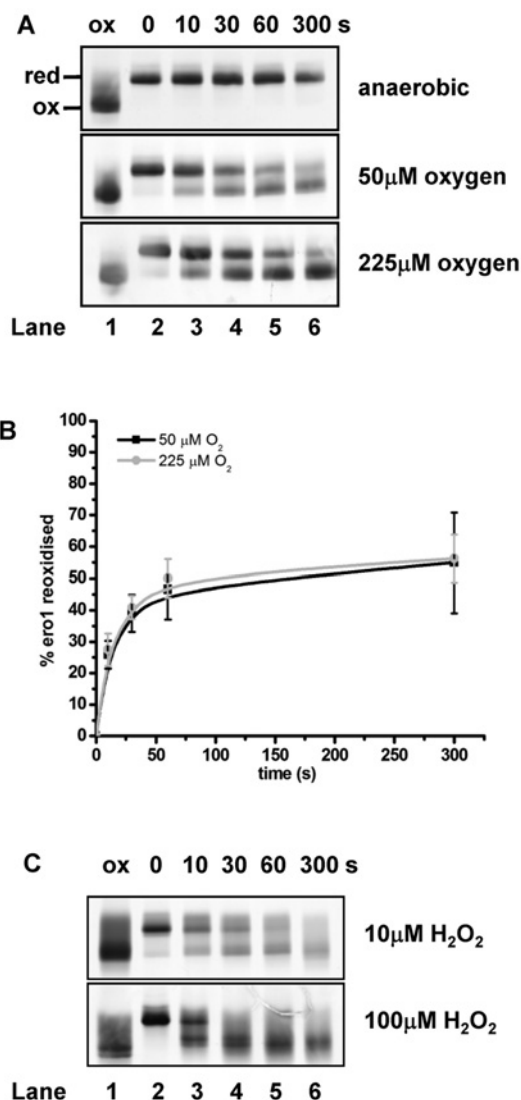
We and others have shown that the redox status of Ero1 $\alpha$  changes during its reaction with thioredoxin, specifically the regulatory disulfides are first reduced during substrate oxidation and then are themselves oxidized [4,5]. This transition in redox status can be followed by changes in the mobility of the protein when separated without prior reduction and these changes have been shown to be due to the reduction of the regulatory disulfides by mutation of individual cysteine residues [5]. The redox transitions can also be followed by AMS modification during the reaction of Ero1 $\alpha$  with thioredoxin under aerobic conditions (Figure 1B, top panel). An initially oxidized Ero1 $\alpha$  quickly becomes reduced, after 10–120 s, in the presence of reduced thioredoxin. This redox state shift coincides with the oxidation



**Figure 1** Oxygen is essential for the oxidation of thioredoxin by Ero1 $\alpha$

Reduced thioredoxin (100  $\mu$ M) was incubated with Ero1 $\alpha$  (2  $\mu$ M) and at the indicated time points (annotated in seconds) samples were alkylated with AMS to freeze the redox state of both enzymes. Samples were then analysed by non-reducing SDS/PAGE and Coomassie Blue stained to visualize thioredoxin (**A**) or silver stained to visualize Ero1 $\alpha$  (**B**). Reduced and oxidized controls are included for comparison. The assay was carried out under aerobic or anaerobic conditions and in the presence or absence of Ero1 $\alpha$  as indicated. FAD (200  $\mu$ M) was also added to the reactions carried out under anaerobic conditions as indicated. The results are representative of three separate experiments.

of thioredoxin (Figure 1A). The Ero1 $\alpha$  redox state then shifts towards a more oxidized state between 600–1800 s, after the oxidation of thioredoxin is complete. It is of interest to note that Ero1 $\alpha$  does not return to its original redox state within the time course of the assay indicating that some disulfides reform relatively slowly. Under anaerobic conditions, the redox state of Ero1 $\alpha$  again displays a shift from oxidized to a reduced form between 0–120 s; however, this form of Ero1 $\alpha$  does not shift towards the more oxidized form at 600–1800 s (Figure 1B, middle panel). Hence once the disulfides are reduced by thioredoxin, they do not reform if no oxygen is present in the reaction buffer. In addition, in the presence of exogenous FAD the redox state of Ero1 $\alpha$  follows a similar pattern to that in its absence, confirming that non-enzyme bound FAD cannot act as an oxidant to regenerate disulfides (Figure 1B, bottom panel). These results clearly demonstrate that, in contrast with the yeast enzyme [11,12], Ero1 $\alpha$  cannot utilize FAD as an alternative electron acceptor.

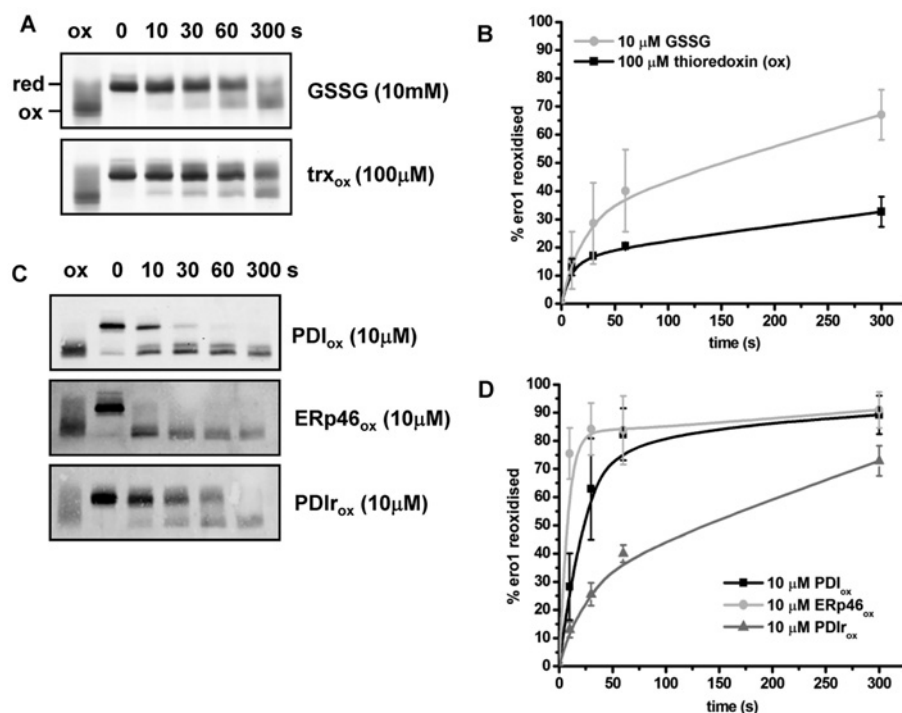


**Figure 2** Autonomous oxidation of Ero1 $\alpha$  by oxygen and H<sub>2</sub>O<sub>2</sub>

Reduced Ero1 $\alpha$  (2  $\mu$ M) was incubated either under anaerobic conditions or with increasing concentrations of oxygenated buffer (**A**) or H<sub>2</sub>O<sub>2</sub> (**C**). The redox state of Ero1 $\alpha$  was frozen by alkylating with NEM at the given time points (annotated in seconds), before analysing the samples by non-reducing SDS/PAGE and silver staining. The positions of the fully reduced (red) and oxidized (ox) forms are as indicated. (**B**) The fraction of Ero1 $\alpha$  re-oxidized in the presence of oxygen for three independent experiments was quantified and the mean value calculated and plotted against the time of incubation (error bars represent S.D.).

### The correct regulatory disulfides within Ero1 $\alpha$ are re-oxidized slowly in the presence of oxygen or H<sub>2</sub>O<sub>2</sub>

Having established that disulfides within Ero1 $\alpha$  once reduced do not reform under anaerobic conditions, we wanted to determine whether oxygen or H<sub>2</sub>O<sub>2</sub> could bring about the reformation of these disulfides. We developed an assay whereby Ero1 $\alpha$  could be stabilized in the reduced state under anaerobic conditions before monitoring its re-oxidation after the re-addition of oxygen to the system. Ero1 $\alpha$  was reduced with DTT under anaerobic conditions before removal of the reducing agent. After the addition of oxygen, the alkylating agent NEM was added at various time points to freeze the redox state and samples were analysed by



**Figure 3** Ero1 $\alpha$  is oxidized rapidly and efficiently by specific ER oxidoreductases

Reduced Ero1 $\alpha$  (2  $\mu$ M) was incubated under anaerobic conditions with (A) either GSSG (10 mM) or oxidized thioredoxin (100  $\mu$ M) or (C) oxidized PDI, Erp46 or PDIr all at 10  $\mu$ M. After the time points indicated (seconds) the redox state of Ero1 $\alpha$  was frozen with NEM and samples analysed by non-reducing SDS/PAGE and silver stain (A) or Western blot with an antibody against Ero1 $\alpha$  (C). ox, oxidized; red, reduced. (B and D) The fraction of Ero1 $\alpha$  re-oxidised for three independent experiments was quantified and the mean value calculated and plotted against the time of incubation (error bars represent S.D.).

SDS/PAGE carried out under non-reducing conditions. There is a distinct difference in the mobility of reduced and oxidized Ero1 $\alpha$  when separated under non-reducing conditions due to the formation of the long-range regulatory disulfides (Figure 2A, lanes 1 and 2) [5]. Ero1 $\alpha$  remained in the reduced state under anaerobic conditions throughout the 300 s time course (Figure 2A, top panel). Introducing 50  $\mu$ M oxygen into the buffer with reduced Ero1 $\alpha$  resulted in the partial oxidation of Ero1 $\alpha$  over 300 s (Figure 2A, middle panel). Increasing the concentration of oxygen in the buffer to 225  $\mu$ M did not further increase the rate or amount of Ero1 $\alpha$  re-oxidation (Figure 2A, bottom panel). The partial oxidation of Ero1 $\alpha$  by oxygen indicates that disulfide exchange within or between Ero1 $\alpha$  molecules can occur albeit inefficiently to re-oxidize the regulatory disulfides.

H<sub>2</sub>O<sub>2</sub> is produced by Ero1 $\alpha$  as a consequence of disulfide bond formation. Hence we determined whether H<sub>2</sub>O<sub>2</sub> may induce sulfenylation of cysteine thiols within Ero1 $\alpha$  leading to regulatory disulfide formation. The redox state of Ero1 $\alpha$  was followed over 300 s following the addition of either 10 or 100  $\mu$ M H<sub>2</sub>O<sub>2</sub>. In the presence of 10  $\mu$ M H<sub>2</sub>O<sub>2</sub>, Ero1 $\alpha$  shifts from the reduced to the oxidized state (Figure 2C, upper panel). Oxidation begins almost immediately and the proportion of oxidized Ero1 $\alpha$  increases until 300 s. The inclusion of 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> results in increased Ero1 $\alpha$  oxidation; however, the bands corresponding to the oxidized form of the enzyme are diffuse and difficult to quantify, suggesting several different disulfide linked products (Figure 2C, lower panel). These results demonstrate that whereas H<sub>2</sub>O<sub>2</sub> may provide a means of Ero1 $\alpha$  oxidation, an ensemble of forms are produced with different combinations of disulfides. Hence it is unlikely

that H<sub>2</sub>O<sub>2</sub> itself leads to the formation of the regulatory disulfides within Ero1 $\alpha$ .

### Ero1 $\alpha$ is oxidized rapidly by specific ER oxidoreductases

The glutathione buffer within the ER along with the PDI family of enzymes could facilitate the formation of the regulatory disulfide bonds within reduced Ero1 $\alpha$ . To determine whether individually these factors could reform the Ero1 $\alpha$  regulatory disulfides we incubated reduced Ero1 $\alpha$ , under anaerobic conditions, with GSSG or various oxidized oxidoreductases. In the presence of 10 mM GSSG and 2  $\mu$ M Ero1 $\alpha$ , the regulatory disulfides were formed relatively slowly and to a limited extent after 300 s (Figure 3A, upper panel). GSSG itself is therefore unlikely to regulate Ero1 $\alpha$  activity. In addition, 100  $\mu$ M oxidized thioredoxin exchanged its disulfide with Ero1 $\alpha$  only to a limited extent (Figure 3A, lower panel) suggesting that this enzyme inefficiently oxidizes the regulatory disulfides in the absence of oxygen. Reduced Ero1 $\alpha$  was also incubated with various ER oxidoreductases, including PDI, Erp46 and PDIr (Figure 3C). These proteins were chosen due to the availability of purified recombinant protein and their known role as good (PDI) or poor (Erp46) substrates for Ero1 $\alpha$  [13]. PDI and Erp46 were able to oxidize Ero1 $\alpha$  regulatory disulfides quickly and efficiently at low molar ratios (2  $\mu$ M Ero1 $\alpha$  and 10  $\mu$ M oxidoreductase) within 10 s and 30 s respectively. PDIr was also able to re-oxidize Ero1 $\alpha$ , but with slower kinetics (Figure 3C, bottom panel). These results demonstrate that oxidized PDI family members are able to

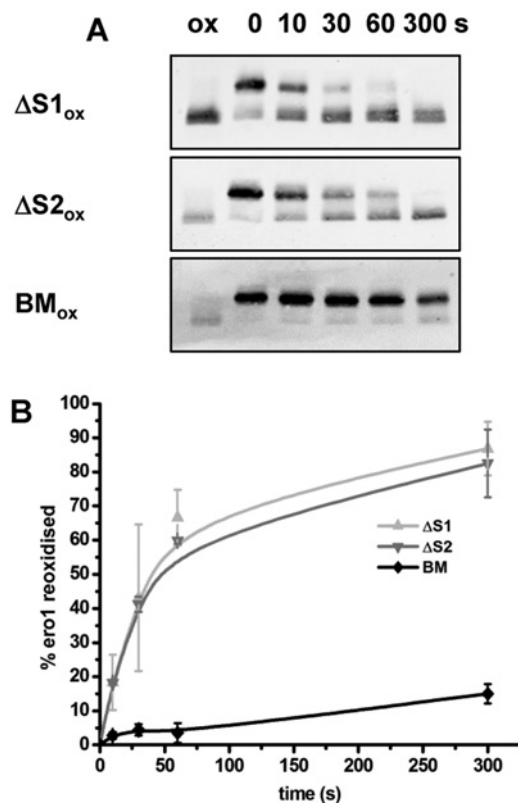
exchange disulfides with Ero1 $\alpha$  in the absence of oxygen resulting in the direct reformation of the regulatory disulfide. The efficiency of the exchange reaction is likely to be dependent on the reduction potential of the disulfide within the thioredoxin domains as oxidized thioredoxin with a relatively low reduction potential was unable to efficiently exchange its disulfide with Ero1 $\alpha$ .

#### Oxidation of Ero1 $\alpha$ by the individual thioredoxin domains of PDI is influenced by the substrate-binding domain of PDI

Previous studies have shown that reduced PDI interacts with active Ero1 $\alpha$  during its oxidation via the PDI substrate-binding domain [6,8,14] and that Ero1 $\alpha$  preferentially oxidizes the PDI a' domain active site [7,8]. To investigate whether a similar interaction between reduced Ero1 $\alpha$  and oxidized PDI is required for the reformation of the regulatory disulfides we determined the ability of three PDI mutants to exchange disulfides with Ero1 $\alpha$ . These were:  $\Delta$ S1, which has the a' domain active site mutated to AGHA;  $\Delta$ S2, which has the a domain active site mutated to AGHA; and BM, which has three point mutations (I272A, D346A and D348A) within the b' domain that prevent substrate binding [15]. Each mutant was assessed for its ability to exchange disulfides with reduced Ero1 $\alpha$  under anaerobic conditions (Figure 4). Using the PDI active site AGHA mutants ensure that any disulfide exchange between PDI and Ero1 $\alpha$  can only be via the remaining intact active site. The  $\Delta$ S1 mutant was able to oxidize Ero1 $\alpha$  at a similar rate to the  $\Delta$ S2 mutant (Figure 4A, top and middle panels). Strikingly, the PDI substrate-binding mutant (BM) was not able to efficiently exchange its disulfides with Ero1 $\alpha$  (Figure 4A, bottom panel). These results suggest that there is no specificity in the ability of the two active site domains of PDI to exchange disulfides with Ero1 $\alpha$ , although the substrate-binding domain is critical to allow the exchange to occur.

#### DISCUSSION

The activity of Ero1 $\alpha$  in mammalian cells is tightly regulated to prevent excessive oxidative stress due to the production of H<sub>2</sub>O<sub>2</sub>. This regulation comes principally from two intramolecular disulfides that, when formed, inhibit Ero1 $\alpha$  activity [4,5]. The Ero1 $\alpha$  substrate, PDI, is able to reduce the regulatory disulfides within Ero1 $\alpha$  in order to activate the enzyme and in doing so regulates the formation of its own active site disulfide [16]. Until recently, it was unknown how these regulatory disulfides reform to inactivate the enzyme or what contribution to this process is made by autonomous oxidation or by enzymatic catalysis. It has been demonstrated that the regulatory disulfides in the yeast enzyme can be reformed by molecular oxygen and that this process can be accelerated by PdiP [10], but it was still an open question as to whether a similar mechanism exists within mammalian cells and whether there is any specificity in the oxidoreductases capable of catalysing this process. The conclusion from the present study is that whereas the regulatory disulfides in Ero1 $\alpha$  can be reformed by oxygen in the absence of an enzymatic catalyst, the rate of reformation is dramatically enhanced in the presence of specific oxidized PDI family members even in the absence of oxygen. The ability of PDI to catalyse the activation and inactivation of its own catalyst provides an elegant mechanism to ensure PDI can fulfil its role as a disulfide exchange protein both introducing and removing disulfides from substrate proteins (see Figure 5). It has been shown that the presence of constitutively active Ero1 $\alpha$  in cells leads to an increase in the amount of oxidized PDI [4], which would compromise the ability of PDI to reduce non-native disulfides in substrates.

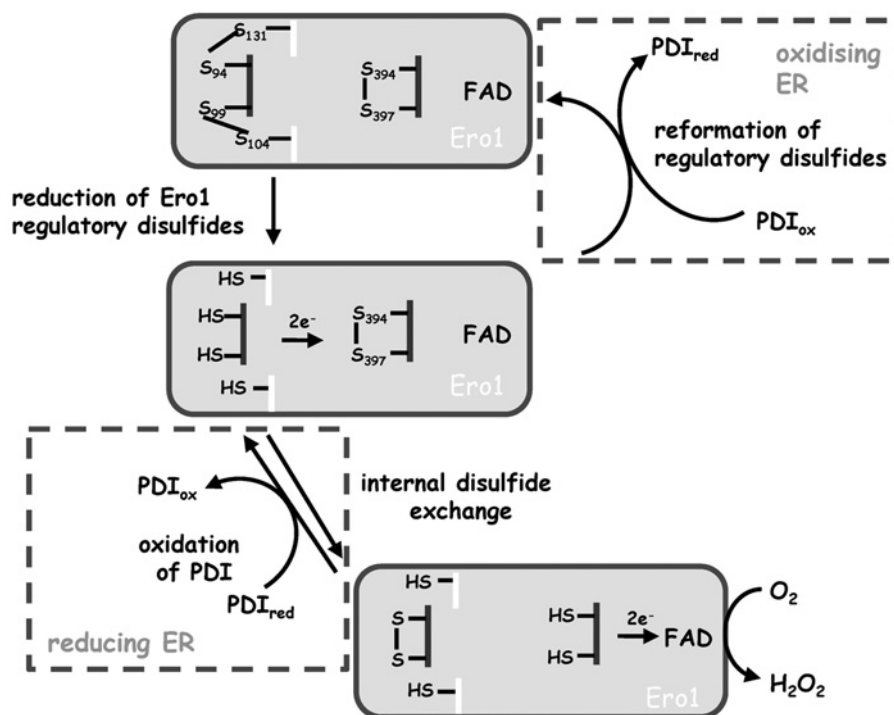


**Figure 4 Both PDI active sites contribute to Ero1 $\alpha$  re-oxidation and inactivation, as does the PDI b'-binding domain**

Reduced Ero1 $\alpha$  (2  $\mu$ M) was incubated under anaerobic conditions with the oxidized (ox) PDI mutants  $\Delta$ S1 or  $\Delta$ S2 or the PDI-binding mutant (BM) all at 10  $\mu$ M. (A) After the time points indicated (seconds) the redox state of Ero1 $\alpha$  was frozen with NEM and samples analysed by non-reducing SDS/PAGE and Western blotting with an antibody against Ero1 $\alpha$ . (B) The fraction of Ero1 $\alpha$  re-oxidized for three independent experiments was quantified and the mean value calculated and plotted against the time of incubation (error bars represent S.D.).

We also show that Ero1 $\alpha$  does not use FAD as an alternate electron acceptor to drive disulfide formation. These results contrast with those showing that FAD can act as an electron acceptor for Ero1p both in an *in vitro* assay [11,12,17] and *in vivo* [18]. The reasons for this difference in the ability of yeast and mammalian Ero1 to utilize exogenous FAD is unclear, but highlights the subtle differences between these two enzymes which also includes the lack of conservation of the regulatory disulfides. However, the regulatory disulfides in both enzymes can be reformed in the presence of oxygen alone which suggests that an internal disulfide rearrangement within the enzyme can result in disulfides being shuttled from the cysteine pair proximal to the isoalloxazine ring of FAD to the shuttle cysteines and finally to the regulatory cysteine pair. The fact that PDI can catalyse the formation of the regulatory disulfides in the absence of oxygen suggests that this disulfide exchange occurs directly with either the active site cysteine pair or directly with the regulatory cysteines.

As H<sub>2</sub>O<sub>2</sub> is produced by Ero1 $\alpha$  as a direct consequence of its activity [17] it was important to determine its ability to reform the regulatory disulfides. Unlike oxygen, H<sub>2</sub>O<sub>2</sub> could directly oxidize the thiol groups to form sulfenylated cysteine which would quickly be resolved by adjacent thiols to form a disulfide. Our results suggest that, whereas H<sub>2</sub>O<sub>2</sub> was able to oxidize reduced thiols in Ero1 $\alpha$ , there was a lack of specificity with several differently disulfide bonded species being formed. It is still an open question what the concentration of H<sub>2</sub>O<sub>2</sub> is in



**Figure 5** Regulation of the Ero1 $\alpha$  redox cycle by PDI oxidoreductases

The activation of Ero1 occurs via a two-step reaction mechanism, namely the reduction of its regulatory disulfide by PDI, followed by an intramolecular disulfide exchange with the inner active site disulfide proximal to the isoalloxazine ring of FAD. Under more reducing ER conditions Ero1 is activated to oxidize PDI. The oxidation of PDI is coupled to the reduction of molecular oxygen to produce  $\text{H}_2\text{O}_2$ . When ER conditions are more oxidizing, oxidized PDI oxidoreductases can catalyse the reformation of the regulatory disulfide bond in Ero1, leading to its inactivation. ox, oxidized; red, reduced.

the ER. Although Ero1 $\alpha$  may well be a source of this molecule there are several peroxidases, such as peroxiredoxin IV [19] and glutathione peroxidase 7 and 8 [20], that are highly active and would be able to rapidly remove any  $\text{H}_2\text{O}_2$  produced by Ero1 $\alpha$ . Hence the lack of specificity in directing disulfide formation and the potentially low concentrations make it unlikely that  $\text{H}_2\text{O}_2$  is involved in the reformation of the regulatory disulfides in the ER.

Previous data have suggested that Ero1 $\alpha$  binds to PDI via a protruding  $\beta$ -hairpin structure which interacts specifically with a binding pocket within the PDI b' domain [6]. In the present study we find that disruption of the binding pocket by mutating three key residues has a similar effect on the ability of PDI to exchange disulfides with Ero1 $\alpha$  to reform the regulatory disulfides. Although the substrate-binding function of PDI is essential for its ability to interact with Ero1 $\alpha$ , Erp46 is able to efficiently exchange disulfides with Ero1 $\alpha$  despite the absence of a similar non-catalytic substrate-binding domain. Previous work has shown that Erp46 is a substrate for Ero1 $\alpha$ , albeit at a much reduced rate compared with PDI [13]. Given the efficiency of inactivation of Ero1 $\alpha$  by oxidized Erp46 it is unlikely that the slow oxidation of Erp46 is due to a lack of physical interaction between these two proteins. Hence the catalytic domains of Erp46 must be able to efficiently interact with Ero1 $\alpha$  to reform the regulatory disulfides circumventing the requirement for a non-catalytic substrate-binding domain. In contrast it may well be the case that the efficient oxidation of PDI family members by Ero1 $\alpha$  requires the presence of a non-catalytic substrate-binding site, a possibility that is supported by the efficient oxidation of Erp57 when its b' domain is replaced with the same domain from PDI [6].

Taken together, the data presented in this study of Ero1 $\alpha$  regulation reveal that, whereas molecular oxygen and  $\text{H}_2\text{O}_2$  can induce disulfide formation within Ero1 $\alpha$ , the enzymatic catalysis of disulfide exchange by specific PDI family members is the most efficient mechanism of activity regulation. The consequence of this tight regulation and the presence of efficient ER peroxidases is that excessive  $\text{H}_2\text{O}_2$  is unlikely to accumulate within the ER of mammalian cells following disulfide formation in mammalian cells.

#### AUTHOR CONTRIBUTION

Colin Shepherd and Neil Bulleid conceived and planned the research. Colin Shepherd performed most of the experiments. Ojore Oka was involved in the purification of the proteins used in the present study. Colin Shepherd and Neil Bulleid analysed the data and wrote the paper.

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